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Differential Regulation of Nitrate Reductase Induction in Roots and Shoots of Cotton Plants[1]

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JOHN W. RADIN
Western Cotton Research Laboratory, Agricultural Research Service, United States Department of Agriculture, Phoenix, Arizona 85040

ABSTRACT

The induction of nitrate reductase activity in root tips of cotton (*Gossypium hirsutum* L.) was regulated by several amino acids and by ammonium. Glycine, glutamine, and asparagine strongly inhibited induction of activity by nitrate and also decreased growth of sterile-cultured roots on a nitrate medium. Methionine, serine, and alanine weakly inhibited induction, and 11 other amino acids had little or no effect. Ammonium also decreased induction in root tips, but was most effective only at pH 7 or higher. The optimum conditions for ammonium regulation of induction were identical to those for growth of sterile-cultured roots on ammonium as the sole nitrogen source. Aspartate and glutamate strongly stimulated induction, but several lines of evidence indicated that the mechanism of this response was different from that elicited by the other amino acids. The effects of amino acids on induction appeared to be independent of nitrate uptake.

In green shoot tissues, all attempts to demonstrate regulation of induction by amino acids failed. The great difference in observed responses of root and shoot to amino acids suggests that their nitrate reductase activities are regulated differently. Differential regulation of this enzyme is consistent with the responses of root and shoot nitrate reductase activity to nitrate.

The enzyme nitrate reductase occupies a control point in the pathway of nitrate assimilation (1). Activity of the enzyme fluctuates widely in response to many environmental or physiological factors, such as the presence of reduced N in the growth medium. The various ways by which enzyme activity can be affected include *de novo* synthesis and turnover (12, 30), allosteric modifications (24, 28), and activation and inactivation (16, 27). In addition, cofactor availability can limit *in vivo* rates of nitrate reduction (5, 13, 18).

Many reports have appeared on the effects of ammonium salts on nitrate reduction. Observations range from a promotion of activity, both with and without nitrate present (11, 23), to inhibition, either by repression or by inactivation (16, 25, 26). In studies of the possible regulation of NR[2] activity by amino acids in higher plants, the conclusions are again contradictory. Filner (7) and Heimer and Filner (8, 9), working with tobacco cell cultures, carefully separated and analyzed the regulation by amino acids of both NR activity and nitrate uptake. They found a complex pattern of control of both activities, including repression and derepression of enzyme synthesis. Stewart (26), working with *Lemna minor*, similarly found amino acid-induced decreases in NR activity that were independent of nitrate uptake. Sims *et al.* (24), also with *L. minor*, showed limited evidence for a slight inhibition of NR, presumably allosteric, by certain amino acids. In contrast, Ingle *et al.* (11), and Schrader and Hageman (23) found no amino acid effects on NR activity in radish cotyledons and corn leaves, respectively. The present paper reports evidence for the regulation of NR by ammonium and amino acids in roots, but not in shoots, of cotton plants.

MATERIALS AND METHODS

Chemicals. L-Amino acids and chloramphenicol were purchased from Calbiochem.[3]

Induction of NR Activity in Roots. Procedures were similar to those described earlier (19). Seeds of cotton (*Gossypium hirsutum* L. cv. 'Deltapine 16') were germinated for 2 to 3 days at 30 C in vermiculite moistened with tap water. Root tips 15 to 20 mm long were excised and transferred to an induction medium on moist filter paper in Petri dishes. The medium contained 100 mM KNO_3, 20 mM glucose, and 10 μg/ml chloramphenicol to retard bacterial growth. In addition, it contained the compounds to be tested and was usually buffered at pH 5.4 with 10 mM phosphate, although the effects of amino acids were little changed by omission of the buffer. Normally, the root tips were induced at room temperature (24 C) for 4 to 5 hr, at which time they contained maximum activity (19).

Sterile Culture of Roots. Seeds were germinated on filter paper in Petri dishes using standard aseptic techniques. After 2 days at 30 C, 10-mm root tips were excised and transferred to 50-ml culture flasks containing 10 ml of sterile liquid medium. The medium contained, in mmoles/l: $CaCl_2$, 1.03; $MgSO_4$, 3.16; KCl, 1.66; KH_2PO_4, 0.15; $MnSO_4$, 0.03; H_3BO_3, 0.024; $ZnSO_4$, 0.0094; KI, 0.0045; Fe ethylenediaminedi-(o-hydroxyphenyl) acetate, 0.0027; Na_2MoO_4, 0.0010; and in mg/l: nicotinic acid, 0.5; thiamin·HCl, 0.1; pyridoxine·HCl, 0.1, and sucrose, 20,000. In addition, the medium contained additions of 3 mM KNO_3, 2 mM $KHCO_3$, 5 mM amino acids, and $(NH_4)_2SO_4$ as necessary. The pH was adjusted to the

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[2] Abbreviation: NR: nitrate reductase.

[3] Mention of a proprietory product does not constitute endorsement by the United States Department of Agriculture.

proper value with 0.1 M HCl or 0.1 M KOH before autoclaving. Each flask contained four or five roots, and was incubated at room temperature on a reciprocal shaker.

Incubation of Leaf Discs. Discs were cut from mature leaves of greenhouse-grown plants as previously described (18). The discs were incubated 4 hr at room temperature in Petri dishes, either in darkness or under fluorescent lamps (6400 lux). The induction medium contained 100 mM KNO_3, 10 mM phosphate buffer, pH 5.4, and the compounds to be tested. After incubation, discs remained turgid and apparently healthy.

Induction of NR Activity in Nitrate-free Plants. Seeds were germinated in a greenhouse for 5 to 7 days in vermiculite moistened with tap water. The green cotyledons were excised just below the node and placed in aerated induction solutions described for incubation of leaf discs. Induction was carried out in darkness at room temperature. After 4 hr, cotyledons were removed from the flasks, blotted, weighed, and assayed.

Assay for NR Activity. Procedures for the *in vivo* assay were described previously (18, 19). In roots, generally only the apical 2-mm sections were assayed. All root-tip assays were run in duplicate or triplicate.

Nitrate Accumulation in Roots. Net accumulation of nitrate in 20-mm root tips was determined after incubation in the standard induction medium either with or without 1 mM Na_2WO_4 present. Duplicate samples of 20 roots were washed with water, weighed, and placed in test tubes containing 10 ml of water. The tubes were capped loosely and heated in a boiling water bath for 30 min, then cooled to room temperature for nitrate analysis. This method, similar to that of Ferrari *et al.* (6), gave better results than homogenization and centrifugation.

Nitrate was determined with a nitrate ion electrode (Orion Research Inc.) and with a Leeds and Northrup model 7413 pH/specific ion meter. Duplicate samples agreed well.

RESULTS

Effects of Amino Acids on NR Induction. Table I summarizes the results of many experiments carried out with excised root tips or leaf discs. Although the NR activity of the controls varied among trials, the effects of the amino acids, expressed as percentages, were reproducible. In root tips, several amino acids affected induction of NR. At 2 mM, glutamine was the most effective inhibitor of induction, followed in order by glycine, asparagine, methionine, serine, and alanine. Eleven amino acids, classified here as neutral, showed mild inhibition or promotion of activity (limits set arbitrarily at 15% deviation from the controls). The difference between alanine, the weakest inhibitor, and the neutral group was poorly marked. Only glutamate and aspartate showed a strong stimulation of activity.

In leaf discs, all amino acids fell into the neutral group defined above (Table I). Since the discs were cut from nitrate-grown plants, the question may be asked whether they can be regarded as equivalent to tissue being induced for the first time, such as the root tips. When the leaves from which the discs were cut received high light intensities in the greenhouse, then the discs were responsive to nitrate in the incubation medium. The standard 100 mM KNO_3 in the medium usually caused a doubling of NR activity, with the higher level of activity maintained until at least 4 hr after excision. Thus, synthesis predominated over loss of NR during incubation. In this respect, the leaf discs were analogous to tissue being induced for the first time. In addition, green cotyledons of plants grown without nitrate also failed to respond to any of the amino acids tested. This presumptive differential regulation

Table I. *Effects of Amino Acids and Ammonium at 2 mM on the Induction of NR Activity in Several Experiments*

Root tips were incubated for 4 hr in 100 mM KNO_3 plus 20 mM glucose, pH 5.4, in darkness. Leaf discs were incubated for 4 hr in 100 mM KNO_3 in the light. All amino acids were not included in every experiment, but each amino acid was tested at least three times in each tissue. Values shown are means of the several trials, expressed as percentage of controls. Control activities varied from 1.23 to 7.06 nmoles $root^{-1}$ hr^{-1} in roots and from 7.21 to 13.26 μmoles (g fresh weight)$^{-1}$ hr^{-1} in leaf discs.

Treatment	NR Activity	
	Root tips	Leaf discs
	% of control	
Gln	42	94
Gly	43	99
Asn	50	115
Met	61	108
Ser	66	112
Ala	75	103
Leu	85	101
Ammonium	89	98
Thr	91	104
Trp	93	96
Lys	94	95
His	94	100
Val	94	100
Ile	96	105
Arg	100	103
Pro	101	104
Tyr	104	102
Phe	107	115
Glu	167	99
Asp	179	114

of NR induction in root tips and in green tissue was investigated further, with emphasis on the inhibitory amino acids glycine, glutamine, and asparagine; the stimulatory amino acids glutamate and aspartate; and ammonium.

Induction in root tips seemed to be more sensitive to glycine than to other amino acids, showing some response to a concentration of 0.1 mM (Fig. 1). Glutamine and asparagine were less effective at low concentration, with threshold levels of 0.3 and 1 mM, respectively (Fig. 1). At these concentrations, which can be considered substrate levels, the possibility cannot be eliminated that the amino acids might simply be converted to some inhibitory metabolite. Although the data presented here do not resolve that question, no inhibitor was found to which the induction was more sensitive. None of the amino acids had any effect on NR activity when they were present only during the assay period. Thus it is likely that their influence was exerted on the induction process, rather than on the enzyme itself or on the machinery of the *in vivo* assay.

Effects of Amino Acids on Nitrate Uptake. Heimer and Filner (8, 9) showed that amino acids can strongly inhibit nitrate uptake and NR activity in cultured tobacco cells. In cotton roots, an amino acid influence on nitrate uptake could have caused the observed effects on NR induction. Accordingly, nitrate accumulation during induction was measured with 1 mM sodium tungstate in the medium. The tungstate decreased NR induction by 78%, presumably by causing the formation of inactive enzyme (10); it therefore minimized the difference between uptake and net accumulation. There were no significant differences in nitrate contents among the amino

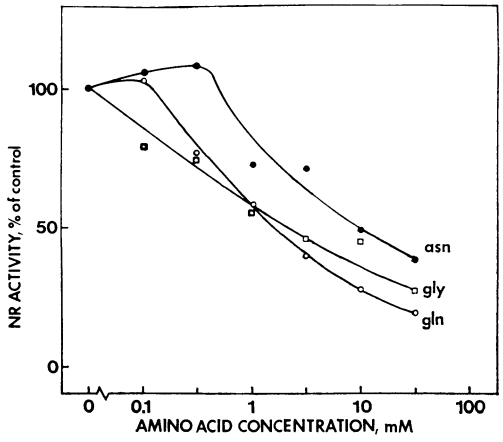

FIG. 1. Effect of amino acid concentration on the induction of NR activity in root tips. Values are expressed as percentage of control (no amino acid). Control values ranged from 2.03 to 5.06 nmoles root^{-1} hr^{-1}. Each point is the mean of two replications.

Table II. *Effects of 5 mM Amino Acids and 1 mM Sodium Tungstate on Net Nitrate Accumulation by Cotton Roots*

Roots were incubated 8 hr in the standard induction solution containing 100 mM nitrate, 20 mM glucose, and chloramphenicol.

Treatment	Nitrate Accumulation		
	+ Tungstate	− Tungstate	Difference
		μmoles/g	
Control	53.9	48.5	5.4
Asn	52.6	48.9	3.7
Gln	53.2	49.0	4.2
Asp	54.7	48.2	6.5
Glu	56.1	48.8	7.3

acid treatments either with or without tungstate (Table II). However, the differences between the nitrate contents with and without tungstate tended to follow NR activities; *i.e.* asparagine- and glutamine-treated roots had smaller differences than the control, and aspartate- and glutamate-treated roots had greater differences than the control (Table II). These results indirectly suggest that amino acids did not influence nitrate uptake, but rather affected net accumulation only through the activity of NR. It is not surprising that uptake from the high solution concentration of 100 mM should be unaffected by amino acids; however, neither were differences in uptake rate among treatments observed with 1 mM nitrate.

Effects of Ammonium on NR Induction. Smith and Thompson (25) clearly demonstrated an effect of ammonium on barley root NR. In cotton roots, ammonium was only slightly inhibitory under the conditions of the standard test (Table I). Subsequently it was found that pH, nitrate concentration, ammonium concentration, and the presence of bicarbonate all strongly affected the response. As the pH was raised from 4 to 8, NR activity declined but the sensitivity to ammonium increased (Fig. 2). In these treatments, bicarbonate at 2 mM was used as a buffer instead of phosphate; preliminary data (not shown) suggest that it also may have stimulated ammonium uptake and metabolism. The effects of pH on NR activity with and without ammonium are consistent with well established effects on nitrate and ammonium uptake, the former decreasing and the latter increasing as the pH is raised (3). Further tests were all conducted at pH 8 with 2 mM bicarbonate as buffer.

There was a marked difference in sensitivity to ammonium in roots induced in the standard test at pH 8 (with 100 mM nitrate), and in roots induced in sterile culture (with 3 mM nitrate). At the higher nitrate level, NR activity was insensitive to ammonium concentrations of 3 mM or below (Fig. 3). At the lower nitrate level, however, roots were sensitive to the lowest ammonium concentration tested, 0.1 mM (Fig. 3). Thus, the levels of both nitrate and ammonium were important in determining the sensitivity of NR induction to inhibition by ammonium.

Stimulation of NR Activity by Glutamate and Aspartate. The stimulation of NR induction by glutamate and aspartate (Table I) was unexpected. However, several aspects of the phenomenon deserve further comment. First, the effect was not uniform throughout the root, with the increase confined largely to the apical 2-mm segment. In contrast, the inhibition by glycine, glutamine, and asparagine occurred approximately equally in apical and subapical 2-mm segments (data not shown). Second, malate and succinate, two other dicarboxylic

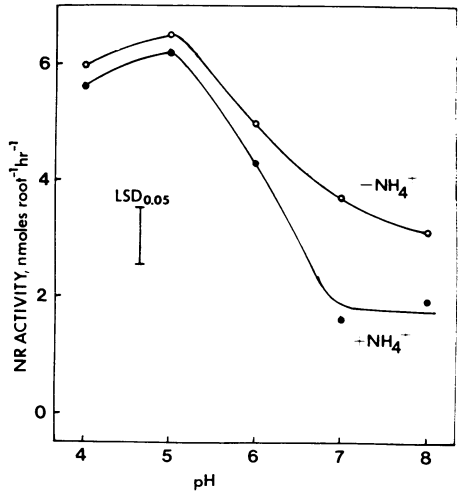

FIG. 2. Effect of pH on induction of NR activity in root tips either with or without 8 mM ammonium. Induction media included 2 mM bicarbonate.

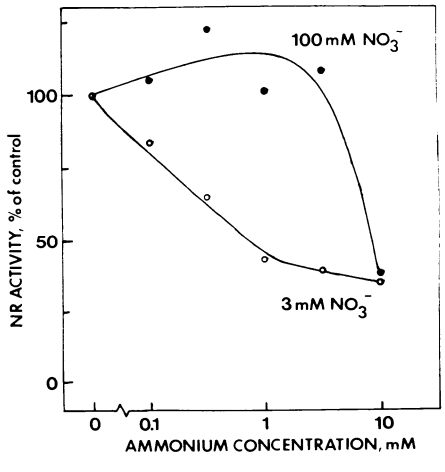

FIG. 3. Effect of ammonium concentration on induction of NR activity at pH 8.0. Bicarbonate at 2 mM was included in all media. Activity was measured in either the standard test system after 4 hr (100 mM nitrate) or in sterile-cultured roots after 16 hr (3 mM nitrate). For details see text. Control rates were 6.32 and 4.95 nmoles root^{-1} hr^{-1} for the high and low nitrate systems, respectively. Each point is the mean of two replications.

acids, also substantially increased activity in root tips. Thus, the mechanism by which NR activity was affected might have been quite different from that of the inhibitory amino acids. Sahulka (21, 22) recently reported a similar effect of aspartate on NR activity in pea roots; in addition, glutamate dehydrogenase activity was greatly increased by either aspartate or glutamate. Since both these enzymes are important in nitrate assimilation, it is possible that they were regulated similarly. It seems unlikely that these compounds were simply being respired through the tricarboxylic acid cycle to produce greater reducing power for the *in vivo* assay, for two reasons: (*a*) the assay was run anaerobically; and (*b*) exogenously applied malate enters a nonmitochondrial pool when taken up and is respired very slowly (15).

Effects of Amino Acids and Ammonium on Root Growth. The suggested roles of the three inhibitory and two stimulatory amino acids were tested with root tips in sterile culture. In roots grown without nitrate in the medium, asparagine and glutamine slightly stimulated growth, whereas glycine was inactive (Table III). In roots grown with 3 mM nitrate, however, all three amino acids strongly inhibited growth. Thus they seemed to be acting specifically on the nitrate assimilation pathway, with no other deleterious effects. These results also point out that the amino acids were, at best, only a partial replacement for nitrate as a nitrogen source.

The two amino acids stimulatory to induction affected roots quite differently from the amides or glycine. In the presence of either aspartate or glutamate, growth was decreased to almost zero regardless of whether nitrate was present (Table III). This is strong evidence for the nonspecific nature of their action, and argues against any physiological role in root growth for increased nitrate reduction effected by their presence. Again, malate had a similar effect.

It should be noted that the induction conditions allowing inhibition by ammonium were identical to those under which roots grew well as ammonium as sole nitrogen source (Table IV). Because ammonium could be assimilated by roots for growth, it is not clear whether its inhibition of NR induction was direct or indirect (*i.e.* through some normal metabolic product resulting from its utilization for growth). These data also demonstrate the beneficial effect of bicarbonate on ammonium-supported root growth at high pH.

Regulation of NR in Green Tissues. Many tests were run on leaf discs from nitrate-grown plants and on cotyledons from seedlings germinated without nitrate. The conditions of these tests included several pH values, light or dark during incubation, and the presence of small amounts of detergent to facilitate entry. No regulation of NR activity was observed at amino acid or ammonium concentrations comparable to those effective on roots. For example, 20 mM ammonium at pH 7.5 had no effect on NR in leaf discs in light or dark, although 100 mM ammonium drastically decreased activity in discs incubated in the light. Presumably this decrease was mediated by uncoupling of photophosphorylation (14). In the nitrogen-deficient cotyledons, 100 mM ammonium increased induction, as shown by earlier investigators (11, 23, 25). Glycine and other amino acids at concentrations up to 30 mM had no discernible effect. Both ammonium and amino acids were readily taken up from solution in either light or dark.

Table III. *Growth Rates of Sterile-cultured Roots in Nutrient Medium Either with or without 3 mM KNO_3 and in the Presence of 5 mM amino acids*

Roots were grown at pH 5.8 for 6 days after transfer. $LSD_{0.05}$: 0.09 mm/hr.

Amino Acid	Root Growth Rate	
	$-NO_3^-$	$+NO_3^-$
	mm/hr	
Control	0.15	0.49
Asn	0.25	0.19
Gln	0.25	0.20
Gly	0.16	0.33
Asp	0.02	0.01
Glu	0.03	0.01

Table IV. *Growth Rates of Sterile-cultured Roots on 3 mM Ammonium as Sole Source of Nitrogen*

The culture media were buffered with either 2 mM bicarbonate or 2 mM phosphate (additional to the amount in the basal medium) or were unbuffered. With an initial pH of 8.0, the decreases in pH during culture were 3.0, 1.0, and 0.9 for the unbuffered, phosphate, and bicarbonate treatments, respectively. Roots were grown for 3 days. $LSD_{0.05}$: 0.25 mm/hr.

In tial pH	Root Growth Rate		
	Unbuffered	Phosphate	Bicarbonate
	mm/hr		
4.0	0	0.04	0.03
5.0	0.36	0.16	0.23
6.0	0.45	0.46	0.44
7.0	0.35	0.48	0.65
8.0	0.32	0.45	0.74

Conclusions from negative results such as these are necessarily tenuous; it may be premature to state that amino acids do not regulate NR induction in green tissues of cotton. However, the difficulty of its demonstration contrasts with the ease and consistency of its demonstration in root tips. It seems reasonable to conclude that regulation in shoots, if it exists, is accomplished differently than in roots.

DISCUSSION

The experiments reported here establish differential regulation of NR induction in roots and shoots of cotton. The mechanism by which differential regulation is accomplished was approached only superficially in this work, and direct evidence probably must await the isolation and characterization of the two NR proteins. It is noteworthy in this regard that, in addition to regulation, other aspects of nitrate metabolism differ in leaves and roots. For example, the intracellular localization of NR is known to be particulate in roots and cytoplasmic in leaves (17, 20). Several possible mechanisms of amino acid action can be eliminated, though, including a direct effect on the machinery of the *in vivo* assay and changes in nitrate uptake rates. In addition, it seems unlikely that ammonium, as a product of amino acid degradation, might mediate the regulation of NR, because roots seemed to be more sensitive to amino acids than to ammonium in the standard induction procedure. Also, if amino acids were degraded to produce ammonium, it was not in large enough quantities to support root growth. The reverse possibility, that newly synthesized amino acids mediate the inhibition by ammonium, seems more likely. However, the strong influence of other factors such as pH makes interpretation of these data difficult.

Differential regulation of root and shoot NR is consistent with accumulated knowledge about the behavior of the

enzyme. Boutard (2) showed that increased nitrate in the medium greatly increased NR activity in shoots of barley, concomitantly with decreases in root NR activity. Wallace and Pate (29) showed the same response to nitrate in field pea. Boutard suggested that root NR might be sensitive to inhibition by endproducts; however, Wallace and Pate postulated that increased nitrate reduction in the shoot simply decreased the supply of carbohydrate available to the root. These suggestions are not incompatible with each other. Differential response to nitrate is also characteristic of cotton plants. Work is continuing to determine whether both mechanisms contribute to the behavior of root NR. Alternatively, it might be argued that induction of root NR activity might simply have a lower optimum nitrate concentration than does that of shoot activity. However, induction in isolated cotton roots is enhanced by increasing nitrate levels up to 100 mM (19).

Low-nitrate plants also characteristically have a low shoot-root ratio (4, 29). Wallace and Pate (29) found that under some conditions root weight declined, as did root NR activity, when the nitrate supply was increased. Again, cotton plants responded similarly. Such a correlation between root NR activity and root growth suggests that root growth may depend more upon root NR than upon shoot NR. The possibility of separate functions for root and shoot NR activities is being pursued in this laboratory.

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